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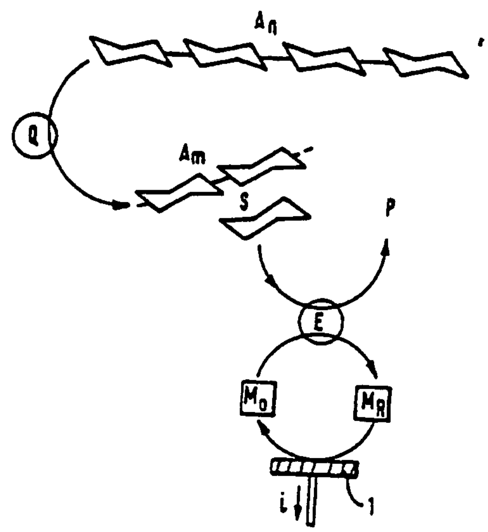
(54) Title: ASSAY FOR DEGRADABLE SUBSTRATES BY ELECTROCHEMICAL DETECTION OF REDOX SPECIES



(57) Abstract

Use of an electrochemical detection of redox species (M_R) in a medium to determine the occurence of a conditi n under which such species are produced or released, wherein such production or release is enabled by the digesti n r disruption of a macromolecular species (A_n). The invention provides a method of assay for a polymer, for a depolymerase enzyme, and for a labelled specific binding agent, in which a polymer (A_n) is labelled with a component of a mediator/enzyme/substrate system which remains inactive until released into solution. Electrodes in the medium determine the occurence of a condition under which redox species are produced by the mediator/enzyme/substrate system or released int solution. Where the mediator activity of, for example ferrocene to glucose oxidase is used an amplification step is pr vided.

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<u>Title</u>: Assay for degradable substrates by electrochemical detection of redox species.

<u>Field of Invention</u>:-

The present invention concerns the electrochemical detection of redox species in a medium and the use of such detection to determine the occurence of a condition under which such species are liberated, produced, sequestered or discharged into a medium.

More particularly, the present invention is concerned with the electrochemistry of mediators in combination with degradable substrates, such as polymers, and with an electrochemical assay which is in one aspect particularly concerned with an assay for the presence of, or amount of, polysaccharide complexes in a sample, but which extends a general assay for detecting the presence of, monitoring the level of or determining the concentration of compounds containing polymeric structures as set forth below and including but not limited to: celluloses, pectins, galactosides, hyaluronic acids, lipids, peptides, nucleic acids and specific binding partners in immunoassay. As a second

limb f the invention, there is disclosed an assay system for enzymes having a polymerase or depolymerase activity, and for other chemical or physical conditions resulting in the making or breaking of chemical bonds, or changes in physical state or condition.

While use may be made of this invention in the chemical industry, especially where complex mixtures are encountered (e.g. in food chemistry or biochemical engineering) it is of particular value in biological investigation and control techniques. More particularly, it lends itself to animal or human medicine, and in particular to <u>in vitro</u> measuring or monitoring of components in body fluids.

<u>Prior Art</u>:-

For convenience, the invention will be described, <u>inter alia</u>, with particular reference to one class of measurements; the determination of the activity of enzymes capable of digesting polysaccharides. Moreover, while the provision of sensors for components in biological fluids is one object of the invention, other and broader objects are not hereby excluded.

For example, it is an object of the invention to provide an improved assay for α-Amylase (1,4-α-D-glucan

glucan hydrolase, E.C.3.2.1.1.), an enzyme which catalyses the hydrolysis of 1,4-α-glycosidic linkages of polysaccharides (e.g. starches and glycogen) and of oligosaccharides to yield maltose and other simple cleavage products.

It is known that the catalytic activity of α-amylase in serum or urine is due to at least two distinct isoenzymes; a first α-amylase from the pancreas and a second from the saliva. This endoenzyme is extensively measured in clinical laboratories for the diagnosis of acute pancreatitis and obstruction of the pancreatic duct. The determination of α-amylase activity is also important for the food industry, especially for starch converting plants, breweries and other fermentation facilities.

The prior art analytical methods for the measurement of α-amylase activity can be classified in three categories, amyloclastic (i.e. starch-iodine), saccharogenic and chromogenic methods, of which the amyloclastic methods represent the majority of routine α-amylase determinations. All of these methods are, however, laborious, costly and require skilled personnel.

It has been shown that the development of enyzme electrodes for the detection of low-molecular weight

products generated by cleaving the polysaccharides can improve the the techniques used in the saccharogenic methods. Yoda and Tsuchida "Chemical Sensors" edited T. Seiyama, K. Fueki, J Shiokawa and S. Suzuki, Proceedings of the International meeting on Chemical Sensors.

Printed proceedings of biosensors meeting in Tokyo pp648-653] have described a polarographic enzyme electrode for the measurement of α-amylase activity based upon the following reaction scheme.

$$\text{starch} \xrightarrow{\alpha\text{-amylase}} + \text{maltose} + \text{maltotriose} + \text{oligosaccharides}$$

$$\text{maltose} + \text{maltotriose} + \text{oligosaccharides} \xrightarrow{\alpha\text{-glucosidase}} \text{glucose}$$

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Hydrogen peroxide produced by the oxidation of glucose by glucose oxidase is determined by a polarographic electrode of the type used in the Yellow Springs Glucose Analyser.

A second polarographic method for determination of α-amylase activity has been described by Scheller and

co-workers [Scheller, Renneberg and Strnad, J. Electroanal. Chem. 194 123-130 (1985)] and is based upon the electrochemical detection of hydrolysis products from modified blue starch, the substrate normally used for chromogenic determination of α-amylase activity. They have used differential pulse polarography at a dropping mercury electrode as the method of detection.

Other ferrocene modified polymers have been described by Simionescu [as mentioned in Sheats, Pittman and Carraher, Chemistry in Britain, August 1984]. Simionescu discusses the attachment of ferrocene carboxylic acid to cellulose and to Polyvinylacetate by ester linkages, and the attachment of ferrocene carboxaldehyde to polyvinyl pyrrolidinone *via* an aldol condensation. It is suggested by Simionescu that the adduct acts as an effective slow-release agent for the treatment of anaemia.

The technical background of the present invention is described in our European Patent Application 82305597 which describes and claims a sensor electrode composed of electrically conductive material and comprising at least at an external surface thereof the combination of an enzyme and a mediator compound which transfers electrons to the electrode when the enzyme is catalytically active.

The purpose of such an electrode is to detect the presence of, measure the amount of and/or monitor the level of one or more selected components capable of undertaking a reaction catalysed by the said enzyme.

Examples of electrode configurations, mediators and uses are given in that and subsequent patent applications by the applicants.

Enzyme/substrate pairs whose electrochemical behaviour in association with mediator compounds have been studied by the Applicants include those listed in Table 1. It is believed that any of these pairs could be utilised in association with a suitable mediator in the present invention, given some limitations on the assay conditions which would be obvious to the man skilled in the art. Of these pairs, it is clearly advantageous to utilise those enzyme/substrate pairs whose behaviour is established in most detail and which give good, preferably linear, response over the expected measurement range.

Table 1:

Enzym Reactions Known To Couple With Mediators

Enzyme	Substrate
Flavo-proteins	
Pyruvate Oxidase	Pyruvates
L-Amino Acid Oxidase	L-Amino Acids
Aldehyde Oxidase	Aldehydes
Xanthine Oxidase	Xanthines
Glucose Oxidase	Glucose
Sarcosine Oxidase	Sarcosine
PQQ Enzymes	
Glucose Dehydrogenase	Glucose
Methanol Dehydrogenase	Methanol and other Alkanols
Methylamine Dehydrogenase	Methylamine
Cytochrome b-linked Enzymes	
Lactate Oxidase	Lactate
Metalloflavoproteins	
Carbon monoxide Oxidoreductase	Carbon Monoxide

In our earlier application, we state that ferrocenes (bis-cyclopentadienyl iron and its derivatives) have advantages over other mediators used with enzyme/substrate reactions for charge-transfer purposes.

Whilst ferrocenes had been found to be of limited value in spectrophotometric assays as a result of their poor solubility in aqueous solution and low extinction coefficients, they have been found to be more suited to a bio-electrochemical system. Ferrocenes have:

(a) a wide range of redox potentials accessible through substitution of the cyclopentadienyl rings which can be functionalised;

(b) electrochemically reversible one-electron redox properties;

(c) a pH-independent redox potential and a slow autoxidation of the reduced form.

Within this general class of ferrocenes, i.e. the monomeric or polymeric derivates substituted around one or both rings, we have found certain individual ferrocenes such as are listed in Table 2.

Table 2.

Some Derivatives of Ferrocene

Ferrocene derivative	E^o	Solubility	ε
1,1'-dimethyl-	100	I,D	-
acetic acid	124	S	370
hydroxyethyl-	161	S	-
ferrocene	165	I,D	335
1,1'bis(hydroxymethyl)-	224	S	385
monocarboxylic acid	275	S	420
1,1'-dicarboxylic acid	395	S	-
chloro-	345	I,D	-
methyl trimethylamino-	400	S	-

S indicates water solubility; I,D mean respectively insoluble, and detergent- solubilised in 3% Tween-20. E^o is in mV vs a standard calomel electrode, ε is measured in $cm^{-1}M^{-1}l^{-1}$.

Other mediator compounds include those listed in Table 3. It should be noted that not all mediator compounds will mediate effectively between a particular redox enzyme and an electrode surface. The selection of the optimum mediator is to some extent determined by the particular enzyme in use, under a given set of chemical and/or physical conditions.

TABLE 3

Mediator Compounds

Other metallocenes,

Ferrocyanide/ Ferricyanide,

Ruthenium Compounds,

Carboranes,

Conductive salts of TCNQ,

Haloanils, and derivatives thereof,

Viologens and their polymers,

Quinones,

Alkyl substituted phenazine derivatives, and,

Bis-cyclopentadienyl $(Cp)_2MX_x$ complexes.

With some of the above mediators it is convenient to employ a solid electrode formed from the compound, while in others the mediator might be coated onto, or provided in solution at, an electrode surface.

Brief Description of Invention:-

In a broad aspect the present invention concerns the use of an electro-chemical detection of redox species in a medium to determine the occurence of a condition under which such species are liberated, produced, released or sequestered, wherein such production or release is enabled by the digestion, build-up or disruption of a macromolecular species

By employing an electrochemical detection system to determine when the liberation, release, sequestration or production of a redox species occurs, and by coupling such liberation, realase, sequestration or production to the digestion, build-up or disruption of a macromolecular species, it is possible to determine the occurence of a number of conditions under which such digestion, build-up or disruption occurs. For the avoidance of doubt, digestion includes the breakage of chemical bonds under a range of conditions, including enzymic digestion of the macromolecular species, build-up includes the polymerisation of a number of monomers, sub-units or oligomers into a macromolecule, and, disruption includes the breakage of bonds by physical processes such as the disruption of lysosomes (including liposomes) or micelles.

According a first aspect of the present invention there is provided an assay system comprising;

a) a macromolecular species comprising at least in part a component, activatable on digestion or disruption of the macromolecular species to produce, liberate, sequester or release directly, or facilitate indirect production, liberation, sequestration or release of redox species within a medium and,

b) electrode means responsive to the presence of the redox species, for electrochemical detection or measurement of the redox species in the medium,

whereby the said detection or measurement is related to the digestion or disruption of the macromolecular species, as enabled, on occurence of a predetermined condition, thereby being capable of a response to the presence type or amount of the macromolecular species, and/or of the said presence or amount of digestion or disruption and/or of circumstances affecting the indirect production of the redox species.

This aspect of the invention provides for the direct or indirect production or release of a redox species following the digestion or disruption of the macromolecular species, and may be employed to determine when conditions occur under which such a macromolecular species is digested or disrupted. For example, it is

possibl to determine the occurence of a condition which causes a macromolecular species composed of a plurality of linked subunits, to separate into individual sub-units, by labelling some or all of the individual components with a redox species. The redox behaviour of the separate labelled sub-units differs from that of the complexed sub-units and separation may be detected thereby. In one form, this aspect of the present invention is seen to comprise a system capable of detecting a redox species in combination with a redox species bound to or held inactive as a component of a macromolecule. On the occurrence of a pre-determined condition which releases the redox compound from its inactive condition or location, a detectable signal is produced.

For example, the redox species may be chemically linked to a solid substrate, or linked to a colloidally or otherwise dispersed, or disolved molecular species inhibiting the redox activity of the species. Such linkage prevents free movement of the redox species and so alters the electrochemical response of the species. By way of further example, the redox species may be chemically linked to a suitably large molecule, to prevent the passage of the redox species across a molecular exclusion membrane or other "molecular filter". Suitable solid substrates include the wall of

an assay vessel, or a suitable insert, such as a region of an electrode other than the working electrode surface or a mass of beads.

Typically however, this aspect of the invention comprises an assay system including; a redox enzyme, a mediator compound capable of facilitating the transfer of charge from the redox enzyme to the electrode, and, a substrate for the redox enzyme; wherein;

a) the component comprises one of said redox enzyme or said mediator, and,

b) the macromolecular species comprises a large-molecule immobiliser material linked to the component,

whereby upon digestion or disruption of the macromolecular species, the redox enzyme, substrate and mediator interact to provide the redox species at the electrode.

In this embodiment, the macromolecular species includes a redox active component which will facilitate the transfer of charge from the enzyme to an electrode, when the component is released from the macromolecule.

Cons quently, the present invention provides method of assay including the steps of;

a) preparing a mixture containing known amounts of;

i) a redox enzyme,

ii) a mediator compound capable of transferring charge from the redox enzyme to an electrode on occurence of a redox reaction, and,

iii) a substrate for the redox reaction,

wherein at least one of components i) or ii) is linked to a macromolecule, whereby charge transfer to the electrode surface is restricted,

b) treating the mixture with an agent suspected of being capable of effectively liberating the component linked to the macromolecule, and,

c) measuring the charge transfer to the electrode surface, whereby the presence of the agent may be determined.

In this method, one of the components i) or ii) is absent from the medium, being linked to the macromolecule. When the assay mixture is treated with a agent capable of libefating the linked component, the enzyme/ mediator/ substrate system is completed, allowing charge transfer to the electrode to produce a detectable signal. In a particular embodiment of this

method, the macromolecular species is a polymer, in which some or all of the monomeric sub-units are labelled with a component of the enzyme/ mediator/ substrate system, and the agent capable of liberating the component can be a depolymerase enzyme suspected of being in the sample.

In those embodiments in which the mediator is bound to the polymer and both the redox enzyme and the substrate of the redox enzyme are present in the assay system, digestion of the polymer by a depolymerase is indicated by an increase in the activity of the mediator.

According to a second aspect of the present invention there is provided an assay system comprising;

a) a reagent system capable of facillitating the production or release of redox species within a medium in the presence of a component produced or released from a macromolecular species by digestion or disruption of the macromolecular species, and,

b) at least one electrode responsive to the presence of the redox species, for electrochemical detection of the redox species in the medium.

In this aspect of the invention, the analyte of the assay system may be the macromolecular species itself, and the assay system is such that in the pres nce of the

macromolecular species, the reagent system produces red x species which can be detected at the electode.

For example, it is possible to digest or disrupt the complex into components some of which are themselves redox species. However it is not necessary for the components to be themselves redox species.

Accordingly the present invention extends to an assay system including either; a redox enzyme or a mediator compound capable of facilitating the transfer of charge from the redox enzyme to the electrode, and a substrate for the redox enzyme; wherein;

a) the component comprises one of said redox enzyme or mediator,

b) the system further comprises a further enzyme to produce or release the component in the presence of the macromolecular species,

wherein on treatment of the assay system with a sample containing the macromolecular species charge is consequently transferred via the mediator compound to the said electrode.

Thus, the component of the redox enzyme/ substrate/ mediator system is released when the macromolecular species is acted upon by the further enzyme.

In an embodiment, the macromolecular species may be a modified polymer, such as a polysaccharide, and the further enzyme may be a polysaccharide depolymerase, such as amyloglucosidase or α-amylase.

The present assay systems may be employed in methods of assay for specific binding reactions, such as in methods of immunoassay.

As an example, the invention provides a method of assay including the steps of;

a) preparing a mixture containing known amounts of;

i) a redox enzyme,

ii) a mediator compound capable of transferring charge between the redox enzyme and an electrode surface on occurence of a redox reaction,

iii) a substrate for the redox reaction, and,

iv) a ligand labelled with an enzyme having a depolymerase activity on a polymer,

wherein, at least one of components i) or ii) is linked to the polymer whereby charge transfer is restricted until the component is liberated into the mixture,

b) treating the mixture with a sample suspected of containing an antiligand capable of a

specific binding reaction with the ligand, and,

c) measuring the pertubation of charge transfer, whereby the presence of the reactive species may be determined.

In this aspect of the invention, there is provided a method suitable for the detection of a specific binding reaction, in which the ligand is labelled with an enzyme having a depolymerase activity. This activity is detected by the liberation from a macromolecular substrate by the enzyme of a component of the redox enzyme/ mediator/ substrate system. When all three parts of this system are present, charge transfer occurs at the electrode.. The extent to which this charge transfer occurs is determined by the activity of the depolymerase, especially in the instance where the product of the depolymerase is a mediator to the redox enzyme. When the ligand becomes involved in a specific binding reaction, the depolymerase activity is affected with a consequent effect on the transfer of charge to the electrode. Thus, the specific binding reaction may be detected indirectly.

As has been mentioned above, and will be described more fully below, the macromolecule can be a polymer molecule, by which term we intend to include homopolymers, copolymers or large-molecular chains with

r lated but different units e.g. protein, other polypeptide-linked substances and nucleic acids. Alternatively, it can be the surface of a solid particle, e.g. a polystyrene bead. In both these cases the breakage of a covalent chemical bond accompanies liberation of the component linked to the immobiliser, either the digestion of the polymer or the cleavage of the component from the immobiliser. As a further alternative liberation of the immobiliser component accompanies the disruption of a large transfer restraining molecule or aggregation of molecules. For example, the immobiliser can comprise a liposome, attackable by a phospholipase.

Although the above aspects of the invention have been generally described in terms of activities which disrupt or digest macromolecules it is possible to reverse the process and consequenty to detect a polymerase activity. Accordingly, the present invention extends to an assay system for an enzyme having a polymerase activity comprising:

a) a quantity of monomer or oligomer capable of being condensed into a polymer,

b) an enzyme having a redox activity on the said monomer or oligomer,

c) a mediator compound capable of transferring charge from the enzyme (b) to an electrode when the

enzyme (b) is catalytically active,

wherein on treatment of the assay system with a sample containing a polymerase capable of polymerising the said oligomers or monomer, the rate of redox activity of the enzyme (b), and consequently the amount of charge transferred via the mediator compound to the said electrode, is reduced by the polymerisation of the monomer or oligomer.

For the avoidance of doubt, the term "depolymerase" is intended to mean any enzyme which is capable of degrading a polymer into monomers or oligomers or a mixture of products. This includes, but is not limited to hydrolase enzymes, peptidases, hyaluronidases, galactosidases, and nucleases. "Polymerase" has the reverse meaning, and includes both those polymerase enzymes which use a template (such as the enzymes involved in RNA and DNA synthesis) and those enzymes which while manufacturing polymers do not require a template.

Previous amperometric techniques have employed the sequestration of the enzyme component of a enzyme/ substrate/ mediator system by means of non-covalent binding forces, such as are found in the interaction of an antibody with antigen, hormone with receptor, magnetic particle with magnetic body or b tween

complementary strands of nucleic acid. The present invention is distinguished from these in that the mediator is generally held by a covalent bond or other immobilising influence.

In those aspects of the invention in which the mediator compound is chemically attached to the polymer, so that its "mediator" or charge transfer ability is reduced or eliminated until the polymer is attacked, it has been found preferable that the mediator is a metallocene, and more preferably ferrocene or a derivative thereof. It should however be noted that other, non-metal, mediators as listed above in Table 3 can also be used, provided that the mediator activity is recovered when the mediator is released from the immobilising large molecule.

Ferrocene and derivatives thereof are particularly suitable as mediators in the present invention, as they have been shown to retain their activity even when considerable modification and/ or substitution of the ferrocene molecule has been performed.

As mentioned above, the depolymerase activity may be that of a hydrolase and particular utility has been found with those embodiments in which the depolymerase is specific to a polysaccharide. The second enzyme is

preferably selected such that it has a a redox activity on the oligo- and mono-saccharide fragments produced by the action of the depolymerase.

Thus in a preferred embodiment of the present invention the redox enzyme is glucose oxidase or glucose dehydrogenase.

In a first preferential embodiment, amyloglucosidase (E.C.3.2.1.3), hydrolyses α-1,4 and α-1,6-glucan linkages in polysaccharides to produce monomers quantitatively. The polymer substrate is a polysaccharide, amylose, which has been modified by covalent attachment of ferrocene via an ester linkage. After digestion of the polymer by the hydrolytic enzyme, the products can mediate between glucose oxidase and an electrode, in the presence of a glucose substrate, and generate a measurable electrical signal.

In a second preferential embodiment, α-amylase (E.C.3.2.1.1), acts on a second ferrocene-labelled insoluble substrate to produce soluble oligomers which can be detected amperometrically. The cleavage products are capable of acting as electron acceptors for glucose oxidase in the enzyme catalysed oxidation of glucose, thus giving rise to an amplification of the observed current.

In both cases polymer-bound ferrocene, is electrochemically silent, that is, it cannot act as an electron acceptor for glucose oxidase and does not exhibit reversible or quasi-reversible electrochemistry.

The invention will be further described by way of example and with reference to the following figures wherein;

Figure 1; shows a schematic drawing of an assay system in which the analyte is a polymer, and in which the substrate of the enzyme from which charge transfer occurs is a monomer or an oligomer of the polymer,

Figure 2; shows an assay system in which the analyte is a depolymerase, and in which a mediator compound is immobilised as a constituent of a polymer until monomers and oligomers having mediator activity are released by the action of the depolymerase,

Figure 3; shows an assay system in which the analyte is a reagent capable of releasing immobilised enzyme into the free portion of the assay system, and in which both substrate and a mediator for that enzyme are present in the free portion,

Figure 4: shows in schematic form a system in which the releas of the m diator compound into the assay medium is accompanied by the cleavage of a specific type of chemical bond.

Figure 5; shows an extension of the technique illustrated in figure 5, in which a molecular exclusion membrane is employed,

Figure 6: shows in schematic form the operation of a assay system in which the electrochemical response of the mediator compound is determined directly,

Figure 7: shows a) DC cyclic voltammogram of 3mg of a ferrocene derivative of a polysaccharide in 1ml K_2HPO_4:NaCl buffer, pH 7.0 @ 37°C,

b) DC cyclic voltammogram of the solution (a) after incubation at 37°C for 10min. with 25U α-amylase, and,

c) as for (b), but with the addition of 40µl of a 1M solution of glucose and 10µl of a 2.3mM solution of glucose oxidase.

Figure 8; shows a) DC cyclic voltammogram of 3mg of a ferrocene derivative of a polysaccharide in 1ml K_2HPO_4:NaCl buffer @ 37°C with 50µl of human saliva, and,

b) as for (a), but with the addition of 40µl of a 1M solution of glucose and 10µl of a 2.3mM solution of glucose oxidase.

Figure 9: Shows a calibration plot of α-amylase concentration, against measured current.

Figure 10: Shows a calibration plot of IgG-α-amylase concentration, against measured current.

<u>Figure 1</u>; <u>Assay for Polymer</u>.

Turning to figure 1, there is shown a schematic diagram of an assay system in which a polymeric species (A_n) is the analyte, and the substrate for an enzyme/mediator system comprises a monomer or oligomer of the polymer (A_n). The polymeric species (A_n) is degraded by a site or component specific depolymerase enzyme (Q) to produce shortened polymers (A_m) and monomers (S) possibly also together with dimers, trimers, etc. as discussed above.

If the structure of the polymer (A_n) is known and the action of the enzyme (Q) is accurately predicted, it is possible to design a system in which either or both of the oligomers (A_m) and the monomer (S) are a substrate for the redox enzyme (E), which is usually an oxidase,

and to which redox enzyme a mediator such as ferrocene is available.

(A_n) in its original polymeric form cannot act as a substrate for (E). Therefore, this system will measure the concentration of digested (A_n) and give an indirect measure of the activity of the depolymerase (Q), in the form of current (i) in the electrode (1). If this activity is known, as is assumed in the present example, a measure of the concentration of the original polymer can be derived.

If any free monomer (S), is pre-existing in the assay mixture, it is possible to perform a corrective assay for the polymer using the differential between the current in the presence and the absence of the depolymerase (Q). It is envisaged that such measurements could be made simultaneously in a system which has more than one sensing electrode and a plurality of compartments separated by suitable molecular-exclusion membranes.

The illustrated system can also be used to assay for the depolymerase (Q), by providing an excess of polymer (A_n) in the assay mixture and omitting the depolymerase (Q). As the polymer is not a substrate of the enzyme (E) no current will be detected at the

electrode until digestion of the polymer (A_n) occurs, thus the presence of the depolymerase (Q) can be detected.

It will be apparent to the man skilled in the art that the above systems can be embodied in competitive assays. For example, if an assay system contains substrate (S), enzyme (E), and mediator, but the depolymerase (Q) is replaced by a polymerase, which has the same substrate (S) as the enzyme (E) and which produces a polymer which is not a substrate for the enzyme (E), then a competitive reaction will occur between the consumption of substrate (S) at the enzyme (E) and the polymerisation of (S) into an inactive species. The current generated by such a system would be changed if any of the rate or concentration parameters, such as activity of polymerase or enzyme (E), or such as availiabillity of substrate (S) were altered.

Figure 2: Assay for Depolymerase.

Figure 2 shows an assay system in which the analyte is a depolymerase (R), and in which a mediator compound is immobilised as a constituent of a polymer (B_n) until monomers and oligomers having mediator activity are released by the action of the depolymerase (R).

In the presence of depolymerase (R), mediator is liberated and is free to tranfer charge from the enzyme E to the electrode (1). It should be noted that the substrate (S) and the product (P) of the enzyme (E) are not determined by the nature of the depolymerase, and consequently a wide range of enzymes, substrates and mediators can be employed with this particular embodiment of the invention. It should also be noted that presence of depolymerase in the assay system for even a short time will cause a permanent increase in the electrode current, as unlike the system illustrated in figure 1, charge transfer will continue if the activity of the depolymerase (R) is terminated.

A variant of this technique is directed at the assay of a further analyte and causes a change in the behavior of the assay system shown in the figure by its effect upon at least one of the rate or concentration parameters, such as activity of depolymerase (R). For example, in a specific modification of this technique, the depolymerase (R) is covalently attached to one or other of a pair of specific binding partners such as antigen/antibody. In this configuration, the depolymerase (R) with antibody attached retains its activity and the system function in an identical fashion to that described above.

This technique could be used to assay antibody concentration or, in sandwich assays or heterogeneous competitive assays, to assay or monitor antigen concentration. This configuration is particularly suitable for the estimation of high molecular-weight antigens.

A further alternative is that the liberation of the immobillised component accompanies the disruption of a large transfer restraining molecule or aggregation of molecules. For example, the immobiliser can comprise a liposome containing and confining therewithin a mediator compound and attackable by a phospholipase to release the mediator into the assay system and to thus allow electron transfer to occur.

Figure 3; Assay for release of Enzyme

In the assay system shown schematically in figure 3 the analyte is a reagent capable of releasing immobilised enzyme (E) into the free portion of the assay system, and in which both substrate (S) and a mediator for enzyme (E) are present in that free portion. The enzyme (E) may be immobilised in several ways. For example, as with the mediator confinement technique set out above, th enzyme (E) can be contained within a liposome (L)

and released by the action of a by a phospholipase (T) on the liposome.

__Figure 4__: __Peptide Hydrolysis__.

Figures 4a and 4b, show a system which comprises an assay for a peptide bond hydrolysing condition. The enzyme (E) may perform its catalytic activity upon the substrate (S), in this instance present in solution.

A mediator (F) is attached via a linker group L_1, to a structure L_2 including a peptide bond P.

Little or no charge is transferred to the electrode, whilst the mediator is confined by the peptide bond (P).

On occurrence of a condition which causes the breakage of the peptide bond P, as shown in figure 4(b) the mediator/L_1 conjugate may freely diffuse and transfer charge from the enzyme to the electrode, thereby giving a detectable signal, and indicating that the peptide bond P has been broken.

__Figure 5__: __Molecular Exclusion Membranes__.

Figure 5 shows an alternative system to that shown in Figure 4 which makes use of a molecular exclusion

membrane (3) to separate the mediator (M) which is bound t a large molecule (X), and the enzyme (E), both of which are too large to pass through the membrane (3). In this example, breakage of the linkage between the mediator (M) and the large molecule allows the mediator to diffuse through the membrane and mediate the transfer of charge from the enzyme E to the electrode.

Figure 6; Redox Enzyme Free Systems

In figure 6 a system is shown which lacks the redox enzyme generally referred to as E in the other figures. The only enzyme which is required in the assay system is that which depolymerises a polymer (C_n) which has mediator attached thereto. As with the system illustrated in figure 2, mediator is liberated by a depolymerase (R). It is possible to obtain a response to the presence of the electrochemically active monomer at the electrode (1), from which the activity of the depolymerase can be determined. It should again be noted that presence of depolymerase (R) in the assay system for even a short time will cause a permanent change in the electrode response.

EXAMPLE 1: Pr paration of 6-O-ferrocene-amylose

In order to perform a method such as that illustrated in figures 2 and 7, 6-O-ferrocene-amylose was prepared as described in Methods in Carbohydrate Chemistry Vol IV p 300 for the preparation of 6-O-tosyl-amylose.

1.5g of amylose was suspended in 30 ml dry pyridine and stirred at 35°C for 30 mins, 517 mg of ferrocenoyl chloride (prepared by the literature method) was then added in one portion and stirring was continued for 1hr. The mixture was then poured in a thin stream to 250ml of an ice cold solution of methanol and water (4:1 v/v). The product precipitated immediately as a bright orange compound. This was collected by centrifugation.

The centrifuged product was washed with twice with each of methanol, water and acetone and finally again with methanol to leave a clean solid and colourless supernatant.

EXAMPLE 2: **Electrochemistry of f rrocene-amylose in a three electrode cell with a graphite working electrode**

D.C. cyclic voltammetry experiments were performed using a two compartment cell with a working volume of 1 ml. In addition to the working electrode, the cell contained a 1 cm^2 platinum gauze counter electrode and a saturated calomel reference electrode. All potentials were referred to the saturated calomel electrode (SCE).

Samples of the insoluble ferrocene-amylose complex were sonicated. The buffer used for electrochemical experiments was 0.1M phosphate buffer (pH 7.0) containing 50mM glucose.

The ferrocene-amylose conjugate (3.0 mg) was suspended in phosphate buffer (10 mls) and sonicated for 2 mins to produce a finely dispersed suspension. After sonication, the suspension was placed in the sample compartment of the electrochemical cell and mixed thoroughly using a magnetic stirrer.

After a suitable equilibration time (10 mins), the d.c. cyclic voltammogram of the suspension was recorded over the potential range 0 to +650 mV vs SCE. The cyclic

voltammogram of th ferrocene-amylose complex suspended in buffer showed no direct electrochemistry at the working electrode.

EXAMPLE 3: Electrochemistry of ferrocene-amylose in the presence of amyloglucosidase

Amyloglucosidase (1 mg) was added to the electrochemical cell and the sample was thoroughly mixed. The cyclic voltammogram of the solution was recorded at 5 and 15 mins after the addition of amyoglucosidase. On addition of amyloglucosidase, electrochemistry due to the release of 6-O-ferrocenoyl glucose from the complex under the action of the enyzme, was observed.

It was apparent that incubation of the ferrocene-amylose complex with amyloglucosidase for longer times results in an increase in the current from the direct electrochemistry of the reaction products.

EXAMPLE 4: Electrochemistry of ferrocene-amylose in the presence of amyloglucosidase, glucose oxidase and glucose.

After recording the cyclic voltammograms in example 3, glucose (50 µl of a 1M solution in acetate buffer, pH 4.8) and glucose oxidase (50 µl of a 3 mg ml^{-1}

solution in acetate buffer, pH 4.8) were added to the suspension and the cyclic voltammogram recorded.

Addition of glucose and glucose oxidase to the electrochemical cell caused an increase in anodic peak current from 110nA to 285nA since the degraded ferrocene-amylose acts as a mediator to the glucose oxidase as illustrated in general terms in figure 2.

It can be seen that ferrocene when attached to a polysaccharide shows no electrochemistry whatsoever at a working electrode until that polysaccharide is enzymatically hydrolysed. The progressive degradation of polymers containing electroactive species by enzymic digestion of liberates these species to exhibit their electroactive properties.

The following examples (5-10) relate to the action of α-amylase in hydrolysing amylose polymer and derivates thereof.

<u>EXAMPLE 5</u>: <u>Preparation of antibodies labelled with α-amylase.</u>

α-amylase was conjugated to bovine IgG using glutaraldehyde. α-amylase (24mg) and IgG (10mg) were dissolved in 2ml phosphate buffer pH 6.8 (0.1M) to this 60μl of a 10% aqueous solution of glutaraldehyde was

added with gentle stirring. The solution was left to stand for 30min. The reaction was terminated by the addition of 100µl of a 1% ethanolamine solution. The conjugate was purified using FPLC ["Fast Protein Liquid Chromatography"; trade-name, of Pharmacia] using a gel-filtration column. The IgG content of the conjugate was assessed using goat-antibovine IgG labelled with alkaline phosphatase.

<u>EXAMPLE 6</u>; <u>Three-electrode system, electrochemistry of 6-O-ferrocene-amylose in the presence of hog-pancreas α-amylase</u>.

When α-amylase was added to the cell to give a final in-cell concentration of 25U, and the contents incubated at 37°C for 10 minutes, a new, quasi-reversible redox couple (E ~ 463mV vs SCE; ΔE_p=125mV) was observed. Figure 7b shows a dc cyclic voltammogram of the solution (a) after incubation at 37°C for 10min. with 25U α-amylase.

The anodic peak current for this couple increased with increasing incubation time and so the species giving rise to this voltammogram can only be from the products of the enzymic reaction.

<u>EXAMPLE 7</u>; <u>Three-electrode system, electrochemistry of 6-O-ferrocene-amylose in the presence of hog pancreas α-amylase, GOD and glucose</u>

Addition of glucose (40μl of a 1M solution in phosphate/NaCl buffer, pH 7.0) and glucose oxidase (10μl of the stock 2.36 mM solution) showed catalytic behaviour with cleavage products. Figure 8c shows a cyclic voltammogram as for example 6, but with the addition of 40μl of a 1M solution of glucose and 10μl of a 2.3mM solution of glucose oxidase.

The catalytic current from the breakdown products was estimated as 3μA at 500mV. It should be noted that in the absence of of α-amylase, the species with a redox peak at 550mV did not show catalytic behaviour when glucose oxidase was added to the solution.

<u>EXAMPLE 8</u>; <u>Three-electrode system, electrochemistry of 6-O-ferrocenoyl-amylose in the presence of salivary α-amylase, GOD and glucose</u>

The above experiment was repeated using 50μl of human saliva instead of α-amylase, resulting in a catalytic current at 550mV of ~2μA. Figure 8a shows a DC cyclic voltammogram of 3mg of a ferrocene derivative of

a polysaccharide in 1ml K_2HPO_4:NaCl buffer @ 37°C with 50µl of human saliva. Figure 8b shows a cyclic voltammogram as shown in figure 8a, but with the addition of 40µl of a 1M solution of glucose and 10µl of a 2.3mM solution of glucose oxidase.

Normal human serum has an α-amylase activity of about 1500 U/l. Therefore the effect of 1 U/ml α-amylase (final concentration) on 3mg amylose-ferrocene polymer was investigated under the conditions described above. The catalytic current measured under these conditions was approximately 90nA.

Example 9: Dose/response curve for α-amylase

Ferrocene-amylose polymer (3 mgs/ml) was sonicated in phosphate buffer pH 7.0 (0.1M) containing 50mM glucose. One ml of this suspension was added to an electrochemical cell, and varying amounts of α-amylase were added. After a 10 minute incubation, excess glucose oxidase was added and the catalytic current measured at +550mV vs SCE a dose/response curve was obtained. The response curve is shown in figure 9.

Example 10: Dose/response curve to antibodies labelled with α-amylase.

The same procedure as described above in example 9, was followed with the sole exception that α-amylase labelled IgG was used instead of α-amylase. The response curve is shown in figure 10.

As can be clearly in Figures 7-9, the action of amylase on 6-O-ferrocene-amylose provides an excellent basis for an amperometric sensor for the measurement of amylase activity. As can be seen from figure 10, the system also provides a label suitable for use with immunoassay systems.

We envisage the use of a two-electrode system to measure both α-amylase activity in serum samples from a normal population as well as those serum samples containing pathologically high levels of α-amylase. This represents a significant improvement over the polarographic method described by Scheller et al.

CLAIMS:-

1) The use of an electrochemical detection of redox species in a medium to determine the occurence of a condition under which such species are produced or released, wherein such production or release is enabled by the digestion or disruption of a macromolecular species

2) An assay system comprising:

a) a macromolecular species comprising at least in part a component, activatable on digestion or disruption of the macromolecular species to produce, liberate, sequester or release directly, or facilitate indirect production, liberation, sequestration or release of redox species within a medium and,

b) electrode means responsive to the presence of the redox species, for electrochemical detection or measurement of the redox species in the medium,

whereby the said detection or measurement is related to the digestion or disruption of the macromolecular species, as enabled, on occurence of a predetermined condition, thereby being capable of a response to the presence type or amount of the macromolecular species, and/or of the said presence or amount of digestion or disruption and/or of circumstances affecting the indirect production of the redox species.

3) An assay system according to claim 2, including; a redox enzyme, a mediator compound capable of facilitating the transfer of charge to or from the redox enzyme to the electrode, and, a substrate for the redox enzyme; wherein;

a) the component comprises one of said redox enzyme or mediator, and,

b) the macromolecular species comprises a large-molecule immobiliser material linked to the component,

whereby upon digestion or disruption of the macromolecular species, the redox enzyme, substrate and mediator interact to provide the redox species at the electrode.

4) An assay system comprising;

a) a reagent system capable of facillitating the production or release of redox species within a medium in the presence of a component produced or released from a macromolecular species by digestion or disruption of the macromolecular species, and,

b) electrode means responsive to the presence of the redox species, for electrochemical detection of the redox species in the medium.

5) An assay system according to claim 4, including either a redox enzyme or a mediator compound capable of facilitating the transfer of charge or from the redox enzyme to the electrode, and a substrate for the redox enzyme; wherein:

a) the component comprises one of said redox enzyme or mediator,

b) the system further comprises a further enzyme to produce or release the component in the presence of the macromolecular species,

wherein on treatment of the assay system with a sample containing the macromolecular species charge is consequently transferred via the mediator compound to or from the said electrode.

6) An assay system according to claim 5, wherein macromolecular species is a redox modified polymer.

7) An assay system for an enzyme having a polymerase activity comprising a quantity of monomer or oligomer capable of being condensed into a polymer, and, covalently linked to said monomer or oligomer, either;

i) an enzyme having a redox activity on the said monomer or oligomer, or

ii) a mediator compound capable of transferring charge to or from the enzyme (i) to an electrode when

the enzyme (i) is catalytically active,

wherein on treatment of the assay system with a sample containing a polymerase capable of polymerising the said oligomers or monomer, the effective amount of the enzyme (i), or the mediator (ii) and consequently the amount of charge transferred via the mediator compound to or from the said electrode, is reduced by the polymerisation of the monomer or oligomer.

8) A method of assay including the steps of:

a) preparing a mixture containing known amounts of:

i) a redox enzyme,

ii) a mediator compound capable of transferring charge from the redox enzyme to an electrode on occurence of a redox reaction, and,

iii) a substrate for the redox reaction,

wherein at least one of components i) or ii) is linked to a macromolecule, whereby charge transfer to the electrode surface is restricted,

b) treating the mixture with an agent suspected of being capable of effectively liberating the component linked to the macromolecule, and,

c) detecting or measuring the charge transfer to or from the electrode surface, whereby the presence or amount of the agent may be determined.

9) A method of assay including the steps of:

a) preparing a mixture containing known amounts of:

i) a redox enzyme,

ii) a mediator compound capable of transferring charge between the redox enzyme and an electrode surface on occurence of a redox reaction,

iii) a substrate for the redox reaction, and,

iv) a ligand labelled with an enzyme having a depolymerase activity on a polymer,

wherein, at least one of components i) or ii) is linked to the polymer whereby charge transfer is restricted until the component is effectively liberated into the mixture,

b) treating the mixture with a sample suspected of containing an antiligand capable of a specific binding reaction with the ligand, and,

c) detecting or measuring the perturbation of charge transfer, whereby the presence or amount of the reactive species may be determined.

10) The assay of claim 3, 5, or 7 or the method of claim 8 or 9 in which the mediator compound is chemically attached to or included within a monomeric unit of a polymer.

11) The assay of claim 3, 5, or 7 or the method of claim 8 or 9 in which the redox enzyme is of the type described as glucose oxidase or glucose dehydrogenase.

12) The assay of claim 6 in which the further enzyme is a deploymerase.

13) The assay of claim 12 in which the deploymerase is amyloglucosidase or α-Amylase.

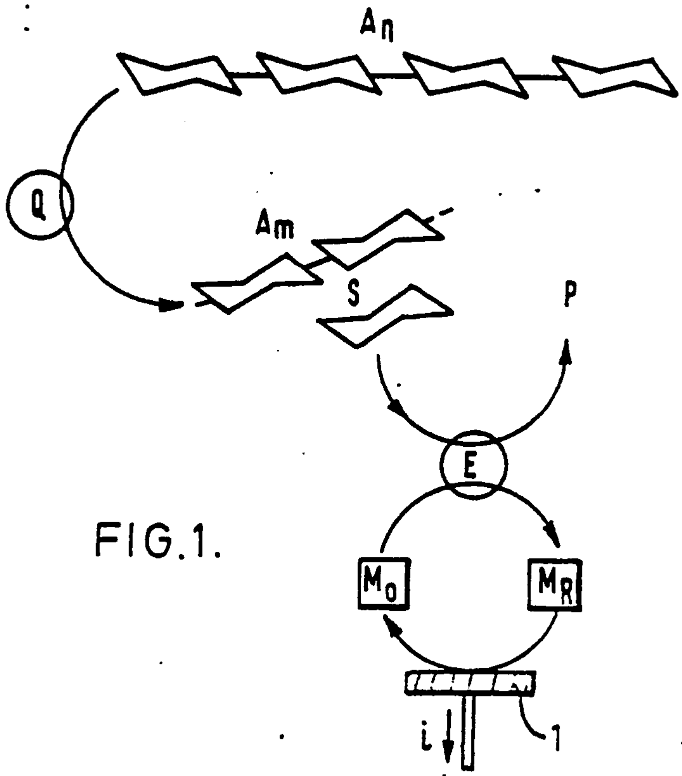

FIG.1.

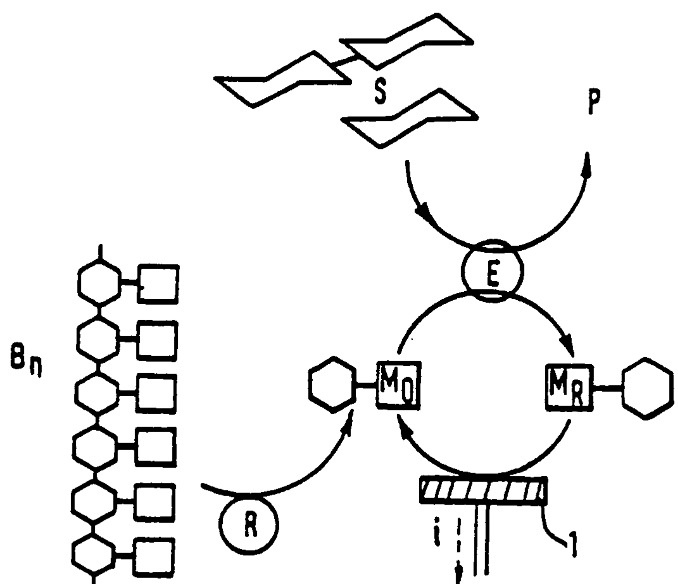

FIG.2.

2/4

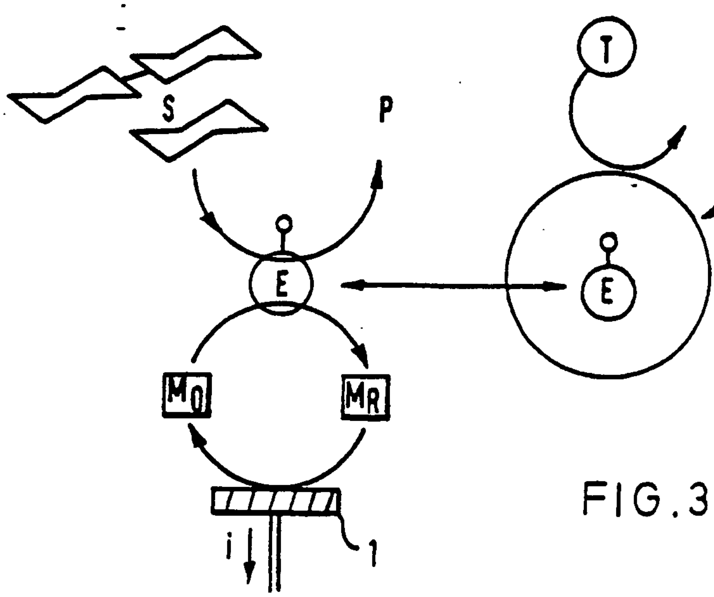

FIG.3.

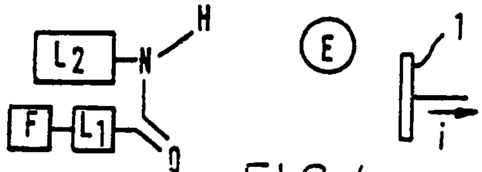

FIG.4a.

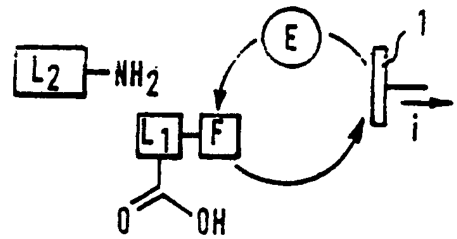

FIG.4b.

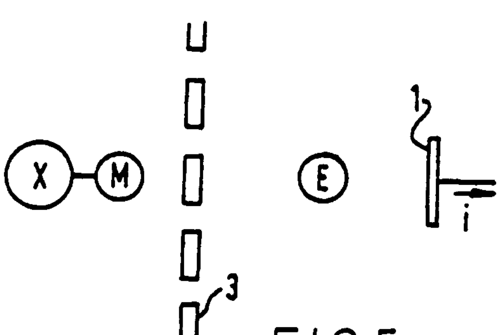

FIG.5.

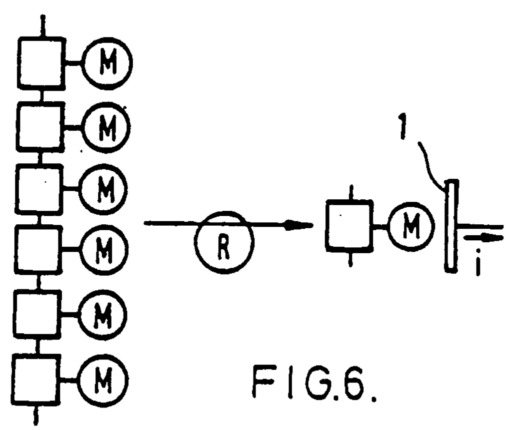

FIG.6.

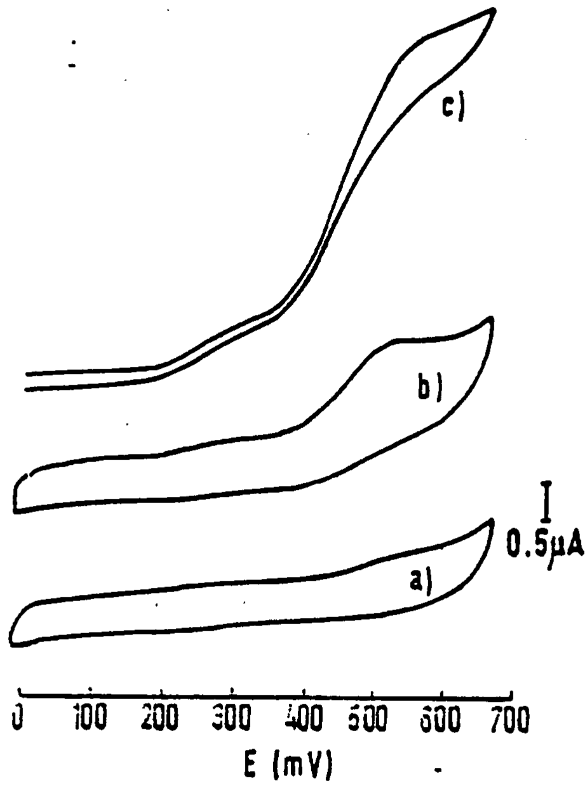
c)
b)
a)
0.5µA
0 100 200 300 400 500 600 700
E (mV)

FIG.7.

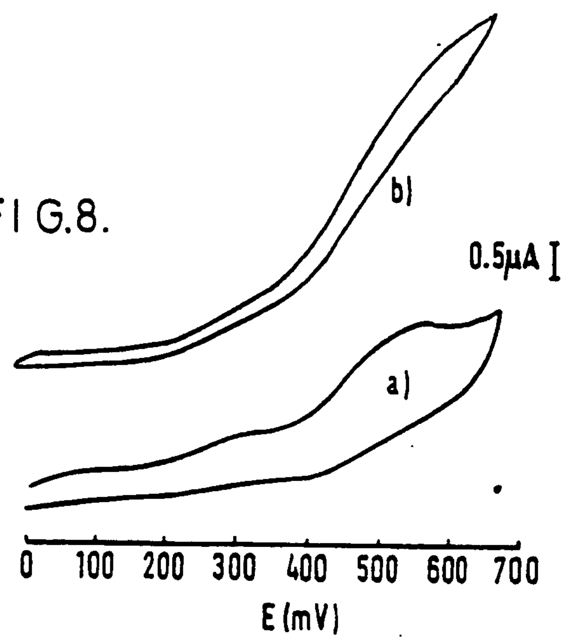
b)
0.5µA
a)
0 100 200 300 400 500 600 700
E(mV)

FIG.8.

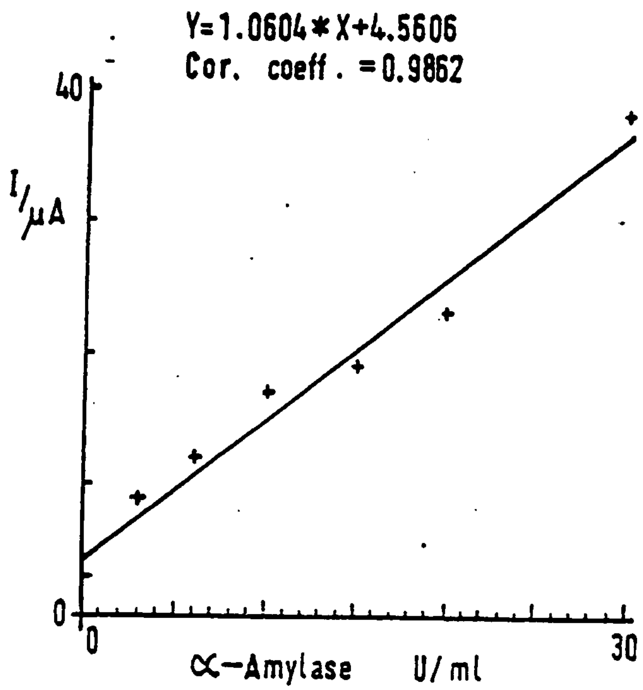
Y=1.0604*X+4.5606
Cor. coeff. =0.9862
40
I/μA
0
0
30
α-Amylase U/ml

FIG.9.

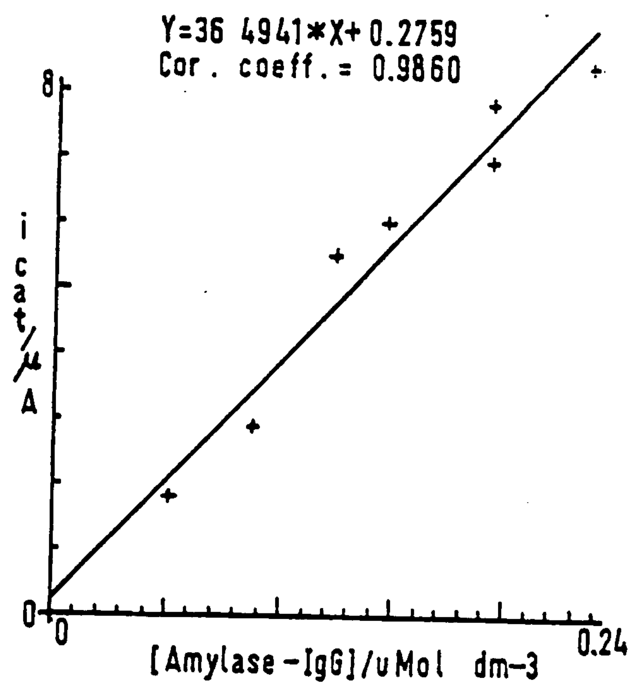
Y=36 4941*X+0.2759
Cor. coeff.= 0.9860
8
icat/μA
0
0
0.24
[Amylase-IgG]/uMol dm-3

FIG.10.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00095

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/00; G 01 N 33/53; C 12 M 1/40; C 12 Q 1/40; C 12 Q 1/28

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q G 01 N C 12 M

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0125136 (GENETICS INTERNATIONAL INC.) 14 November 1984, see abstract; figure 4; pages 19,20; claims 1-17	1-13
P,X, Y	EP, A, 0149339 (GENETICS INTERNATIONAL, INC.) 24 July 1985, see abstract; examples 1,2; figure 1; claims 1-35	1-13
P,Y	EP, A, 0142301 (SERONO DIAGNOSTICS LTD.) 22 May 1985, see the whole document	1-13
P,Y	EP, A, 0150602 (GENETICS INTERNATIONAL, INC.) 7 August 1985, see abstract; claims 1,12-16	1-13
A	EP, A, 0034122 (PENTAPHARM AG) 19 August 1981, see abstract	1,6
A	EP, A, 0089626 (G.D. SEARLE & CO.) 28 September 1983, see abstract; claims 18,20	1,6

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* Special categories of cited documents: [10]

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th May 1986	27 JUN 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/GB 86/00095

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4233403 (R. MENSON et al.) 11 November 1980, see abstract; claim 1	1,6

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00095 (SA 12241)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/06/86

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0125136	14/11/84	EP-A- 0125137	14/11/84
		EP-A- 0125139	14/11/84
		EP-A- 0125867	21/11/84
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		AU-A- 2775484	08/11/84
		JP-A- 60017345	29/01/85
		JP-A- 60017346	29/01/85
		JP-A- 60017347	29/01/85
		JP-A- 60017360	29/01/85
		AU-A- 2775184	31/01/85
		AU-A- 2775284	31/01/85
		EP-A- 0127958	12/12/84
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		WO-A- 8502627	20/06/85
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		CA-A- 1161432	31/01/84
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		US-A- 4480030	30/10/84
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		US-A- 4532207	30/07/85
		CA-A- 1195627	22/10/85
US-A- 4233403	11/11/80	None	

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82